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**Acknowledgements**—The authors wish to thank Prof. H. Bartelink for his continuous support, Dr F.A. Stewart for her fruitful comments and Dr E. Damen for his advice with the SPECT analyses. Mrs M. Langendijk is acknowledged for skilful performing of the iothalamate/hippuran clearances. This study was partially financially supported by the Dutch Cancer Society (Koningin Wilhelmina Fonds), grant NKI 90-15.

*Eur J Cancer*, Vol. 29A, No. 16, pp. 2243-2248, 1993.  
Printed in Great Britain

0959-8049/93 \$6.00 + 0.00  
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# Evidence of Post-translational Regulation of P-Glycoprotein Associated with the Expression of a Distinctive Multiple Drug-resistant Phenotype in Chinese Hamster Ovary Cells

Siobhán McClean and Bridget T. Hill

Chinese hamster ovary (CHO) cells following exposure to fractionated X-irradiation *in vitro* dominantly expressed a distinctive multiple drug-resistant phenotype, characterised by resistance to vinca alkaloids, epipodophyllotoxins and colchicine, but not to anthracyclines, together with overexpression of P-glycoprotein (Pgp), but without any concomitant elevation in Pgp mRNA (*J Natl Cancer Inst* 1990, 82, 607-612; 1992, 85, 48-53). To investigate the mechanism of this Pgp overexpression, Pgp stability was examined in an X-irradiation pretreated subline and compared with that of two colchicine-selected drug-resistant CHO sublines. These studies revealed a slower turnover of Pgp in the X-irradiated cells ( $T_{1/2} \geq 40$  h) relative to the drug-selected sublines ( $T_{1/2} = 17$  h), indicating that Pgp overexpression appears to be differently regulated in these independently-derived resistant sublines. These data add support to our proposal that the development of drug resistance following X-irradiation may arise by a mechanism distinct from that operating after drug selection.

*Eur J Cancer*, Vol. 29A, No. 16, pp. 2243-2248, 1993.

## INTRODUCTION

THE DEVELOPMENT of drug resistance in patients treated with chemotherapy alone or combined with radiotherapy represents a major obstacle to the successful treatment of cancer [1, 2]. *In vitro* multidrug resistance (MDR) is characterised by resistance to many functionally and structurally varied antitumour agents, and overexpression of the 170 kDa membrane protein, P-glycoprotein (Pgp) [3, 4]. Pgp expression is considered to be regulated by a number of mechanisms, including gene amplification [5], transcriptional activation [6] and translational or post-

translational modifications [7]. For example, Chinese hamster ovary (CHO) cells, selected clonally with increasing concentrations of colchicine, exhibited an increase in Pgp gene copy number, Pgp mRNA and protein, at every step in the selection procedure [5, 8]. In contrast, Shen *et al.* [6] observed that in colchicine-selected human KB leukaemia sublines, elevation in Pgp mRNA could occur prior to *mdr1* gene amplification. In a series of human ovarian SKOV-3 carcinoma cell lines, derived by selection with increasing concentrations of vincristine or vinblastine, initially Pgp mRNA and Pgp levels increased without any amplification of the *mdr1* gene, but at higher levels of selection, amplification of the DNA occurred, followed at a later stage by increased overexpression of Pgp without any further increase in Pgp mRNA [7].

Recently, we reported that the expression of a stable multiple

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Revised 29 July 1993; accepted 5 Aug. 1993.

drug resistance phenotype involving Pgp overexpression was not exclusive to drug selection, since CHO cells exposed to fractionated X-irradiation *in vitro* proved resistant to vinca alkaloids, epidophyllotoxins and colchicine [9]. However, these X-irradiated sublines, designated DXR-10I and DXR-10II, which dominantly expressed the multiple drug resistance phenotype [10], retained sensitivity to anthracyclines, and whilst they stably overexpressed Pgp, this was not matched by any significant concomitant elevation in Pgp mRNA [9]. These data imply that either translational or post-translational modifications of Pgp might be involved in overexpression of this protein following X-ray exposure. Therefore, the turnover of Pgp in the DXR-10II cells was examined to investigate whether any differences could be identified, relative to two colchicine-selected resistant CHO cell lines, which showed increased Pgp levels and concomitant elevations in Pgp mRNA [5]. Estimations of the stability of Pgp in the drug-resistant CHO cells were carried out by following the loss of  $^{35}\text{S}$ -labelled Pgp with time, by quantitative immunoprecipitation utilising the C219 or C494 Pgp-related monoclonal antibodies and subsequent analysis on SDS-PAGE gels.

## MATERIALS AND METHODS

### Chemicals and antitumour agents

Protease inhibitors were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). [ $^{35}\text{S}$ ]Methionine (1000 Ci/mmol) was purchased from Amersham International (Buckinghamshire, U.K.).

### Cell lines

The parental AuxB1 cell line, the X-ray pretreated subline, DXR-10II [9] and two colchicine-selected sublines CH<sup>R</sup>A3 and CH<sup>R</sup>C5 [8] were maintained continuously in  $\alpha$  minimal essential medium ( $\alpha$ MEM) plus 10% fetal calf serum (FCS) (both purchased from Gibco Life Technologies, Perth, U.K.), as previously described [9].

### Metabolic labelling of Pgp *in vitro*

Logarithmically growing cells ( $2 \times 10^7$ ) were preincubated in methionine-free (met-)  $\alpha$ MEM plus 10% dialysed FCS. Cells were labelled for 18 h with [ $^{35}\text{S}$ ]methionine (20  $\mu\text{Ci}/\text{ml}$ ) and washed four times with phosphate buffered saline (PBS), before replacing with  $\alpha$ MEM plus 10% FCS. For the "zero time", cells were harvested immediately after washing, while the remaining flasks were incubated at 37°C for periods up to 40 h.

### Immunoprecipitation of Pgp from CHO sublines

The immunoprecipitation procedure was based on that of Anderson and Blobel [11]. Briefly, cells were harvested, washed with PBS and lysed in 200  $\mu\text{l}$  lysis buffer (1% SDS, 0.05 mol/l Tris-HCl, pH 7.4) containing protease inhibitors (4  $\mu\text{g}/\text{ml}$  pepstatin A, 30  $\mu\text{mol}/\text{l}$  leupeptin and 1  $\mu\text{g}/\text{ml}$  aprotinin) by sonification (three  $\times$  3 s pulses at 50 W. Labsonic 1510, FT. Scientific Instruments Ltd, Camberly, Surrey, U.K.). Cells lysates were diluted to 1 ml with 1.25% Triton X-100 (TX-100), 190 mmol/l NaCl in 0.05 mol/l Tris-HCl pH 7.4. Following centrifugation, the lysates were incubated with C219 (Centocor, CIS U.K. Ltd., High Wycombe, U.K.) or C494 (kindly donated by Dr V. Ling, Ontario Cancer Institute, Toronto, Canada) antibodies (10  $\mu\text{g}/\text{ml}$ ) overnight at 4°C. Following recentrifugation, the supernatants were incubated with preconditioned protein A sepharose beads (Pharmacia LKB Biosystems, Milton Keynes, U.K.) for 15 min at room temperature and then for

105 min at 4°C. The immunoprecipitates were washed 5 times with 0.03% SDS, 0.1% TX-100, 150 mmol/l NaCl, 5 ml bovine serum albumin in 0.05 mol/l Tris-HCl, containing protease inhibitors, before a final wash in 150 mmol/l NaCl, 0.05 mol/l Tris-HCl. The immunoprecipitated proteins were eluted from the protein A sepharose beads with 10  $\mu\text{l}$  of sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.05 mol/l Tris-HCl, pH 7.0) and analysed by SDS-PAGE according to the method of Fairbanks [12]. A volume equivalent to 10 000 cpm in the zero control was loaded on gels at each time point, thus standardising the counts loaded. The gels were fixed, dried under a vacuum and the  $^{35}\text{S}$ -labelled protein monitored by fluorography. In order to enhance visualisation of immunoprecipitated Pgp from DXR-10II cells,  $^{125}\text{I}$  radioactivity labelled CH<sup>R</sup>C5 cells were added immediately prior to the cell lysis step to act as a Pgp carrier, and to reduce the specific binding of other cellular proteins to the monoclonal antibodies and to protein A sepharose. This significantly improved the resultant autoradiographs permitting their analysis by densitometry. For the purpose of comparison, additional unlabelled CH<sup>R</sup>C5 cells was also incorporated during immunoprecipitation of Pgp from CH<sup>R</sup>A3 and CH<sup>R</sup>C5 cells, and procedure did not affect the observed half-life.

### Densitometric analysis

Autoradiographs were analysed using an LKB laser densitometer with LKB software. The quantity of protein was calculated as a percentage of control (time zero). At least three autoradiographs of different exposure times were examined each independent experiment to ensure linearity of the readings and the results were expressed as the mean of three independent experiments. Half-life was calculated at the time taken for Pgp to be reduced to 50% of the quantity recorded at time zero and again was expressed as the mean of three experiments  $\pm$  S.E.M.

### Cytotoxicity studies

The relative resistance of CH<sup>R</sup>A3 cells was determined colony forming assay. Logarithmically growing cells were exposed to a range of concentrations of vincristine, etoposide, doxorubicin or colchicine for 24 h before being plated out in 0.17% agarose in  $\alpha$ MEM plus 10% FCS. Colonies were counted 10–14 days later, as described previously [9].

## RESULTS

### Turnover of Pgp in drug-resistant CHO cells determined using the C219 antibody

Significant turnover of Pgp in CH<sup>R</sup>C5 cells over the 40 h period of study was clearly identified and, by densitometry of autoradiographs of three individual experiments, the half-life of Pgp expressed in CH<sup>R</sup>C5 cells was determined as 17 h  $\pm$  3 (Fig. 1.). However, SDS-PAGE gel electrophoresis provided evidence of a slower rate of turnover of Pgp in the DXR-10 cells (Fig. 2) and by densitometry of autoradiographs, the half-life was determined to be  $\geq$  40 h. Immunoprecipitation of Pgp from DXR-10II cells proved more difficult than that from other cell lines examined (compare Fig. 2 with Figs 1 and 3) since considerably less Pgp (20- to 50-fold) was detected in the irradiated cells [9, 10]. Therefore, autoradiography requires longer exposure times which increased the background. An attempt was made to measure the half-life of Pgp in the AuxB1 parental cells, however, the 'baseline' Pgp level in these cells, although just detectable by western blotting [10], proved to be

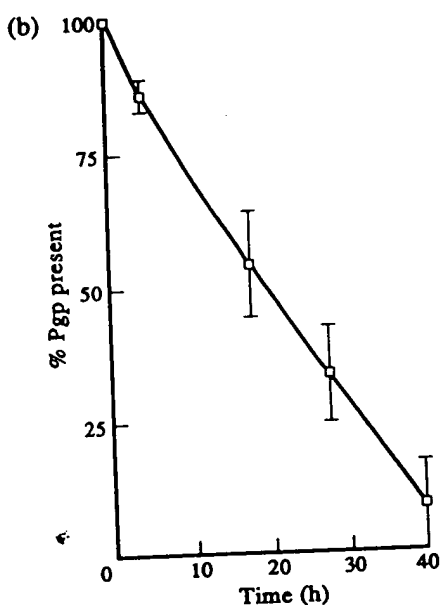
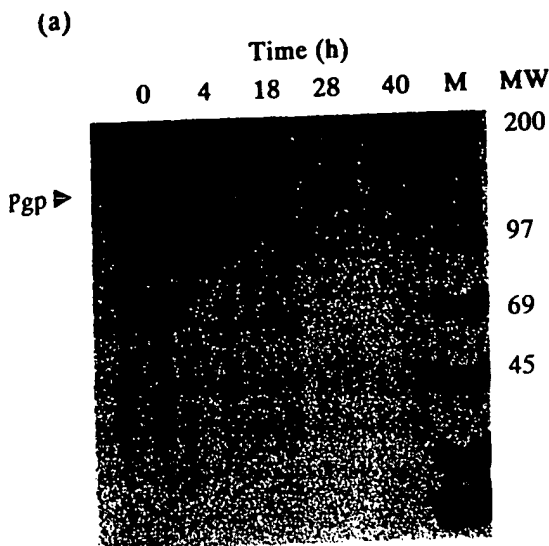


Fig. 1. Turnover of P-gp in CHRC5 cells as determined by immunoprecipitation of  $^{35}$ S-labelled Pgp with C219 monoclonal antibody at different times. (a) Sample autoradiograph; (b) plot of Pgp densitometry readings against time derived from three individual experiments. M, Marker.

too low to permit detection by this method of immunoprecipitation, with no 170 kDa band identifiable. Therefore, although the half-life of Pgp in these DXR-10II cells could not be compared directly with that in the wild type AuxB1 cell line, it appears that the overexpression of Pgp identified in the DXR-10II cells might be explained in terms of a significantly increased half-life relative to that detected in the classic MDR CHRC5 cells.

It is, however, also possible that these dissimilar turnover rates of Pgp might be associated with the markedly different levels of drug resistance expressed by the DXR-10II and CHRC5 cells (i.e. 8- and 120-fold resistant to vincristine, respectively [9]). To overcome this, we chose the CHRA3 cell line for further studies, which was selected in a single step from AuxB1 with 100 ng/ml colchicine [13], and amplified the Pgp gene 10- to 20-fold on Southern blots [5]. By a comparison of  $IC_{50}$  values (the

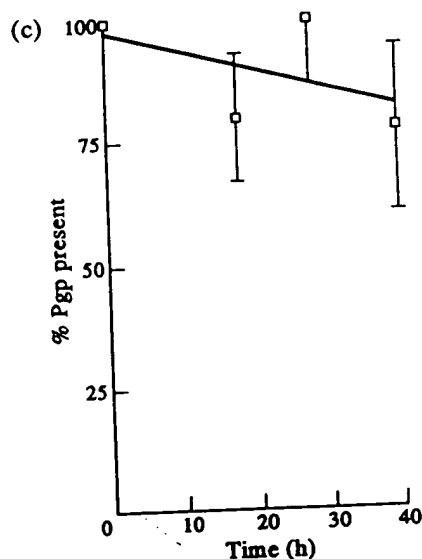
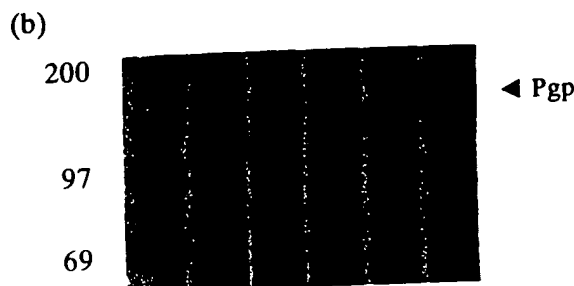
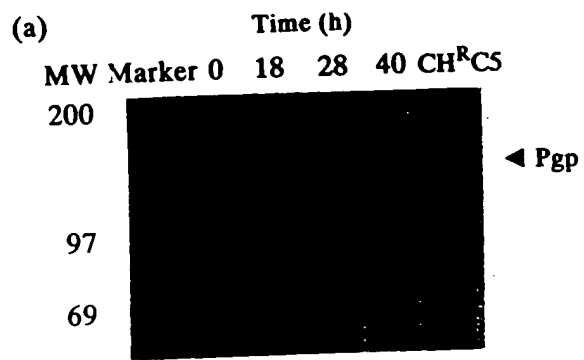


Fig. 2. Turnover of P-gp in DXR-10II cells as determined by immunoprecipitation of  $^{35}$ S-labelled Pgp with C219 monoclonal antibody over a 40-h period. (a, b) Sample autoradiographs; (c) plot of turnover of Pgp with time as determined by densitometry derived from three individual experiments, error bars represent S.E.M.

drug concentration required to reduce cell survival to 50% untreated controls) determined from full dose-response curves these CHRA3 cells proved 6-fold resistant to colchicine, consistent with earlier reports [8], and 3.4-fold resistant to vincristine, 4.4-fold resistant to etoposide and 2.5-fold resistant to doxorubicin. These cells, therefore, provided a useful control for monitoring Pgp stability, in terms of relative drug resistance levels similar to those expressed by the DXR-10II cells. Turnover of Pgp in these CHRA3 cells was found to be rapid ( $10 \pm 1$  h) for Pgp levels to reach 50% of controls, Fig. 3) and certainly comparable with that identified in the CHRC5 cells, rather than the much longer half-life observed in the DXR-10II cells.

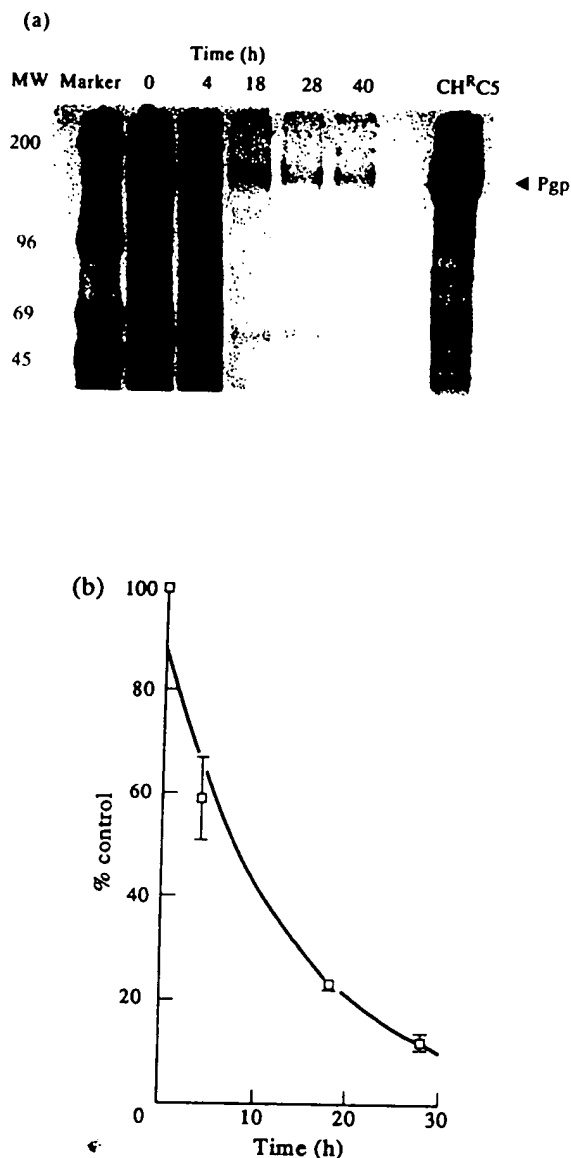


Fig. 3. Turnover of Pgp in CH<sup>R</sup>A3 cells as described by immunoprecipitation of <sup>35</sup>S-labelled Pgp with C219 monoclonal antibody over a 40-h period. (a) Sample autoradiograph; (b) plot of mean densitometry readings against time derived from two individual experiments. Error bars represent S.E.M.

#### Confirmation of enhanced stability of Pgp in DXR-10II cells using the C494 monoclonal antibody

In order to achieve appropriate resolution of Pgp in <sup>35</sup>S-labelled DXR-10II cells, exposures of autoradiographs were required which were longer (up to 10 days) than the 3-day exposures with the CH<sup>R</sup>C5 cells. This had the effect of increasing the resolution of various background proteins, which were expressed in the DXR-10II cells at a higher level than Pgp and were unavoidably immunoprecipitated on the autoradiographs (compare Figs 1 and 2). The monoclonal antibody C494 which recognises a different epitope on Pgp from the C219 monoclonal antibody [14] was used to confirm that the protein which was resolved at 170 kDa in the DXR-10II cells, with a half-life  $\geq 40$  h, was indeed Pgp. Pgp overexpression has been observed in the DXR-10II cells (and CH<sup>R</sup>C5 cells) using this C494 monoclonal antibody [15].

Using C494 for immunoprecipitation of Pgp, it was confirmed

that the Pgp expressed in these DXR-10II cells had a half life  $\geq 40$  h (Fig. 4a). Indeed, the clarity of the 170 kDa band indicated that the C494 monoclonal antibody proved to be a more suitable antibody for immunoprecipitation than C219 in these experiments. As a 'positive' control, turnover of Pgp in CH<sup>R</sup>C5 cells was also estimated using C494 antibody, and again found to be of the order of 17 h (Fig. 4b).

#### DISCUSSION

One of the most distinctive characteristics of the DXR-10II cells was the lack of elevation of Pgp mRNA associated with their Pgp overexpression [9]. These present results demonstrate that this was due, at least in part, to an increased stability of the protein. It has been shown that while the half-life of Pgp was of the order of 10–17 h in drug-selected resistant CHO cells, irrespective of their levels of drug-resistance, Pgp in X-ray-pretreated DXR-10II cells had a half-life of at least 40 h. Since the DXR-10II cells have been found to stably express their distinctive MDR phenotype, in terms of drug resistance levels, Pgp expression and lack of Pgp mRNA elevation, this alteration in Pgp half-life is likely to be a stable change. In agreement with our data, Muller and Ling [16], who examined Pgp half-life in several exponentially growing cell lines using the C219 antibody, including CH<sup>R</sup>C5 and two human ovarian cell lines SKVCR 0.25 and SKVCR 2.0, found that the half-life of Pgp ranged from 12 to 20 h. Recently, we have also estimated the half-life of Pgp in the SKVCR 0.25 cells to be 16 h (unpublished data). The half-life of Pgp in two vinblastine-selected resistant murine sublines has been reported as  $16.8 \pm 0.5$  and  $17.4 \pm 0.5$  h in J7.V1-1 and J7.V3-1 cells, respectively [14], which again correlates well with the findings in the drug-selected CHO sublines examined in this study. In contrast, Pgp turnover has been reported to be slow in MDR human KB cell lines [17, 18] with half-lives of  $> 24$  and 68 h, respectively. However, the extremely high quantity of [<sup>35</sup>S]methionine used in these latter studies (i.e. 250–300  $\mu$ Ci/ml) may have arrested cell proliferation to a certain extent and influenced turnover. Indeed, Muller and Ling in their preliminary report [16] stated that the half-life of Pgp was very dependent on the growth conditions of the cells under study, and could increase up to 72 h when CHO cells were deprived of serum and growth arrested. In the experiments outlined here, care was taken to ensure that all the cells were in logarithmic growth throughout the 40 h period of the study, and it should be emphasised that the population doubling times of all three cell lines examined in this study were comparable, i.e.  $18 \pm 2$  h.

The means by which X-irradiation causes increased expression of Pgp is uncertain and requires further investigation. However, the finding that irradiation of cells can alter the stability of a protein has precedence. It has been reported that normal p53, the tumour suppressor gene product [19], accumulated when non-transformed mouse cells were irradiated with either ultraviolet (uv) or with gamma irradiation [20, 21]. Following uv irradiation, this accumulation was found to be mediated by a post-translational stabilisation, with the half-life increasing from 35 to more than 150 min [20]. It was suggested that the post-translational stabilisation in these 3T3 cells was due to protein-protein interactions between p53 and another nuclear protein, since earlier studies had demonstrated that stabilisation of p53 in SV40 transformed cells was due to a close association of p53 with SV40-encoded large tumour antigen [22].

It is also possible that the rate of synthesis of Pgp may be altered in these DXR-10 cells, relative to the AuxB1 or CH<sup>R</sup>C5

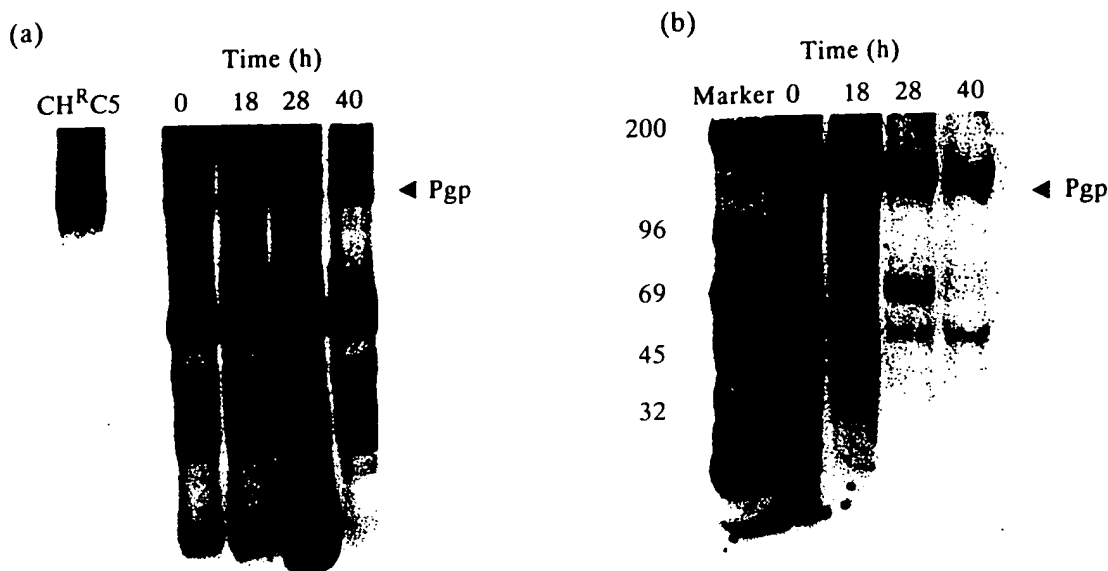


Fig. 4. Turnover of Pgp in (a) DXR-10II and (b) CHRC5 cells as determined by immunoprecipitation of  $^{35}\text{S}$ -labelled Pgp with C494 monoclonal antibody and protein A sepharose.

cells, although current techniques are not sufficiently sensitive to examine rates of Pgp synthesis in these cells. The specificity of this effects in terms of any other proteins which may show altered regulation in the DXR-10II cells, is unknown. However, future studies will aim to address this point. Although glutathione-S-transferase activities have been found to increase by 2-fold in these DXR-10II cells [9], other proteins which have been examined to date, including protein kinase A, protein kinase C (unpublished data) and sorcin [15], have not shown any alterations in expression which would be indicative of a change in regulation. In addition, the finding that an enhancement of Pgp stability is observed in cells which do not show doxorubicin resistance may suggest that these two characteristics are not linked. One possibility is that Pgp expressed by the DXR-10 cells is mutated in some way. The observed increase ability to immunoprecipitate Pgp with C494 from these cells while no increase in clarity of immunoprecipitates from CHRC5 cells was obtained with this antibody (Fig. 4) may reflect this point. Alternatively, other cellular proteins may interact with Pgp in the DXR-10II cells, stabilising Pgp and obscuring the doxorubicin binding site. Both of these possibilities will be examined in future experiments. Meanwhile, the observed increase in Pgp stability found in the DXR-10II cells provides a substantial explanation for Pgp overexpression in the absence of any elevation in mRNA in X-ray pretreated CHO cells. Regulation of Pgp expression is clearly complex, being mediated via at least four different levels involving gene amplification, mRNA overexpression and translational or post-translational modifications [7]. To our knowledge, these findings are the first detailed demonstration of Pgp regulation at the level of post-translational stability. In addition, this report provides further evidence that at least one of the mechanisms by which multiple drug resistance develops in tumour cells, following exposure to X-irradiation *in vitro*, is distinctive from that operating after drug selection.

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- Acknowledgements**—We thank Victor Ling for helpful discussions regarding immunoprecipitation procedures and are grateful to Mary Wallace for her skilled secretarial assistance.

*Eur J Cancer*, Vol. 29A, No. 16, pp. 2248-2250, 1993.  
Printed in Great Britain

0959-8049/93 \$6.00 + 0.00  
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# Inability of Serum Neuron-specific Enolase to Predict Disease Extent in Small Cell Lung Cancer

Elisabeth Quoix, Anne Charloux, Elisabeth Popin and Gabrielle Pauli

Serum neuron-specific enolase (NSE) levels were measured before treatment in 112 patients diagnosed as having small cell lung cancer in our department. All these patients underwent exhaustive staging procedures: 53 had limited disease (LD) and 59 extensive disease (ED). Serum NSE was elevated in 83% of the patients (i.e. 71% of the patients with LD and 93% of the patients with ED). Mean values of NSE differed significantly according to disease extent. A receiver-operating characteristic curve was constructed with different cut-off levels of serum NSE in order to determine the accuracy of NSE for identifying ED. There was no level of NSE capable of predicting with sufficient accuracy the presence of ED. The best compromise was given by a threshold of 35 µg/l: 60% of the ED patients had a serum NSE above 35 µg/l but 30% of the LD patients also had a serum NSE above 35 µg/l.

*Eur J Cancer*, Vol. 29A, No. 16, pp. 2248-2250, 1993.

## INTRODUCTION

PROGNOSIS in small cell lung cancer (SCLC) with respect to response rate and survival is strongly related to the extent of disease [1].

The current two-category staging system (limited disease and extensive disease) requires complex initial staging procedures which are time consuming and unpleasant for these patients. Although some authors [2, 3] have found that the disease stage could be replaced by a more simple prognostic index based on laboratory parameters and performance status, Rawson and Peto [1] could not find a model omitting disease stage that could act as a useful substitute. Moreover, mediastinal irradiation combined with chemotherapy improves local control and overall survival outcome in limited stage disease [4]. Therefore, selection of appropriate therapy requires staging of the patients.

Serum neuron-specific enolase (NSE) is an established useful diagnostic marker for all tumours originating from neuroendocrine cells such as SCLC. Elevated serum levels of NSE have been found in SCLC [5-9] with a sensitivity between 65 and 79%. Specificity is high, since 82-86% of non-small cell lung cancer (NSCLC) have serum NSE levels within normal limits [5, 6].

Consistently higher levels of serum NSE have been found in patients with extensive disease (ED) compared to those with

limited disease (LD) [5-10]. As could be expected, survival also correlated with the initial level of NSE [6, 11].

The aim of our study was to determine if the initial serum NSE level could predict the disease stage (LD versus ED) with sufficient accuracy.

## PATIENTS AND METHODS

During a 4.5-year period (15 October 1987-15 April 1992), 157 patients were diagnosed as SCLC in our department. Among these, a pretherapeutic measurement of serum NSE was performed in 112 (100 males, 12 females). The average age was 58 years (range 37-84).

Initial staging procedures performed in all patients were physical examination, chest X-ray, computerised tomographic (CT) scan of the thorax, bronchoscopy, abdominal ultrasound and/or CT scan, radionuclide imaging study of the bones and/or unilateral bone marrow biopsy, and CT scan of the brain.

All patients were staged according to the standardised classification. LD stage was defined as a disease limited to one hemithorax, including the mediastinal nodes, ipsi- and/or contralateral supraclavicular nodes, and ipsilateral pleural effusion. ED stage was defined as disease beyond that described above. In addition to this classification, tumour size (T) and mediastinal node involvement (N) were assessed.

Determination of serum NSE was done by radioimmunoassay (Pharmacia, Uppsala, Sweden). According to the manufacturer, normal values are below 12.5 µg/l in 96% of the controls.

Serum samples were obtained prior to any treatment.

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Revised 21 Apr. 1993; accepted 11 June 1993.